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Tolerance or Hypersensitivity to 2,4-dinitro-1-fluorobenzene: The Role of Langerhans Cell Density within Epidermis

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Epidermal Langerhans cells have been implicated in the process by which animals skin painted with highly reactive haptens, such as DNFB, develop contact hypersensitivity. Compared to normal body wall skin, murine tail skin contains relatively few, unevenly distributed Langerhans cells; ultraviolet light exposure depletes the epidermis transiently of normal numbers of morphologically identifiable Langerhans cells. When mice are painted with DNFB on skin naturally or artificially depleted of Langerhans cells, contact hypersensitivity is not induced. More importantly, these animals become specifically unresponsive to the chemical contact, and are unable to mount effective hypersensitivity reactions if presented subsequently with an immunogenic regimen. It is concluded that Langerhans cells provide the skin with an intricate dendritic network just beneath the keratinized layer, the function of which is to receive, process and present cutaneously applied antigens in an immunogenic form. When this barrier network is breached, the host responds to antigenic exposure by becoming profoundly and specifically unresponsive. Implications of this hypothesis for epidermal virus infections and cutaneous malignancy are discussed.

The precise manner by which the immunologic apparatus responds to antigen is dependent upon a number of factors of which route of antigen presentation is important. When small molecular weight, highly reactive substances that cause contact hypersensitivity are considered, route of initial antigen presentation becomes crucial. The application of a potential contactant directly to the skin induces a strong state of specific immunologic reactivity. When similar amounts of antigen are ingested by mouth, or inoculated intravenously or intraperitoneally, no comparable state of contact hypersensitivity results. Instead, exposure to simple chemicals (haptens) through noncutaneous routes frequently results in specific unresponsiveness such that treated individuals are specifically *unable* to respond to the eliciting hapten [1–3]. To the skin biologist as well as the immunologist, it is of some importance to understand the reason(s) for this unique and special property of skin.

Macher and Chase [4, 5] proved through a series of elegant, tedious experiments that sensitization following skin painting with hapten required an initial phase during which the hapten reacted directly with a constituent(s) of the skin. Although subsequent processing of this immunogenic signal took place in draining lymph nodes and beyond, the initial, molecular interaction between hapten and a component(s) of skin was essential. It is now known that genes within the major histocompatibility complex (*H-2*) of the mouse exert profound control over the sensitization process [6]. Vadas et al have shown that the development and successful adoptive transfer of delayed and/or contact hypersensitivity to DNFB [7], and fowl gamma globulin [8], is restricted by genes of *H-2* regions *K, I-A, D* and *I-A*, respectively. Shelley and Juhlin [9] have demonstrated that haptenic molecules exposed to the epidermis accumulate preferentially within Langerhans cells.

Over the past 5 yr, the functional properties of Langerhans cells, especially in relation to immune responses, have been the object of considerable investigation [10]. At the present, these curious cells, located suprabasally throughout the epidermis but ontogenetically unrelated to epithelium or neural crest anlage, resemble macrophages-monocytes in several remarkable ways [11]. In addition to their surface affinity for exogenous antigen, they can process and present antigen as effectively *in vitro* to primed T lymphocytes as do conventional macrophages [12]. Langerhans cells have been seen beneath the dermis and along relevant lymphatic drainage routes to regional lymph nodes in the context of delayed cutaneous hypersensitivity reactions [13]. It has been suggested, but not proven, that they derive ultimately from cells within the hematopoietic bone marrow and may be replenished from that source during adult life [14]. It has recently been shown, from several different laboratories, that Langerhans cells are the only cells within the epidermis capable of expressing cell surface determinants encoded by I region determinants of *H-2* [15]. Finally, Langerhans cells have been included as components of a hypothetical "reticuloepithelial system" [16] devised to bind and process antigen applied to the skin and to preside over the dissemination of this immunogenic signal to central compartments of the immunologic apparatus for differentiation into effector function.

If Langerhans cells are in fact the "rock" upon which development of sensitization through skin rests, then skin deficient in these cells might be expected to be unable to sustain sensitization to reactive molecules painted thereon. In the studies to be reported, we have taken advantage of 2 observations to study this question: (1) skin that has been treated with ultraviolet light becomes depleted transiently of Langerhans cells [17]; and (2) the tail skin of mice is naturally deficient in Langerhans cells when compared to the density of these cells in normal body wall skin [17, 18].

MATERIALS AND METHODS

Animals

C57BL/6 mice obtained originally from the Jackson Laboratory, Bar Harbor, Maine, were bred and maintained in our colony. Two to 4-mo-old mice were age matched for each experiment and received pelleted food and water *ad lib.*

Antigens: 2,4-Dinitro-1-fluorobenzene (DNFB) and 4-ethoxynethylene-2-phenyloxazol-5-one (Oxazalone) were obtained from Sigma Chemical Company, St. Louis, Mo.

Sensitization and elicitation of contact sensitivity: Sites through which mice were sensitized were normal abdominal and dorsal body wall skin, UVL-treated abdominal wall skin and tail skin. Mice were sensitized to DNFB by placing 25 μl of 0.5% DNFB in 4:1 acetone-olive oil on the shaved abdominal skin of recipients on day 0 and day 1. Sensitization with Oxazalone was done by placing 25 μl of 10% Oxazalone in the above vehicle on the abdomen on day 0 and day 1. For elicitation of contact sensitivity the ear swelling assay employing painting with the appropriate antigen on day 5 was used. Ear thickness was quantitated using a Mitatoya engineers micrometer as described by

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Abbreviations:
DNFB: 2,4-dinitro-1-fluorobenzene

Phanuphak, Moorhead, and Claman [3]. The dorsal surface of the ears was then challenged with either 20 μl of 0.2% DNFB in 4:1 acetone-olive oil or 20 μl of 1% oxazalone in the same vehicle. 24 and 48 hr after antigen challenge, the degree of ear thickness was again measured and results expressed in units of 10^{-4} inches.

Positive controls consisted of normal mice who were sensitized on body wall skin in the manner described. Negative controls consisted of unsensitized mice that were ear challenged with the appropriate antigen.

Each panel—experimental, positive control, negative control—consisted of at least 5 age- and sex-matched animals. Only 24 hr readings are presented in the figures.

Statistical analysis

The statistical significance of differences in the means for each experimental group was calculated with Students *t*-test. Mean differences were considered significant when $p < 0.05$.

Ultraviolet Light Treatment Source and Treatment

Ultraviolet light (UVL) was administered with a bank of 3 FS-20 "Sun Lamp" fluorescent tubes (Westinghouse, Pittsburgh, Pa.). These tubes provide a continuous ultraviolet spectrum with a peak at 313 nm and high output in the sunburn spectrum (290–320 nm). Tube to target distance measured 45 cm. The minimal erythemal dose on untanned, human skin was 4.0 min, representing an output within the erythema range of 8×10^2 mJ/cm^2 sec.

Langerhans cells were depleted with UV light directed at a 2.5 cm by 2.5 cm area of previously shaved abdominal skin on each mouse. This site was exposed for 2 min on each of 4 successive days (days—3, −2, −1, 0). This dose represented 0.5 minimal erythemal dose in untanned human skin, or 10 mJ/cm^2 each day.

EXPERIMENTS AND RESULTS

Does Ultraviolet Light Treated-Skin Permit Sensitization to DNFB?

We as well as others have shown that ultraviolet light (UVL), in the sunburn spectrum markedly affects the Langerhans cell population within the skin. Four treatments of mouse skin with 2 min of UVL exposure at daily intervals results in the virtual disappearance of ATPase positive cells from the epidermis [17]. During the next 15 days, Langerhans cells gradually return to normal numbers and morphology. The abdominal skin of panels of mice was shaved and treated in this manner with either UVL or incandescent light. Following the 4th treatment, exposed skin of these mice was painted twice at 24 hr intervals with 25 μl of 0.5% DNFB in carrier. Five days later each group of mice was challenged on the ear with 20 μl of 0.2% DNFB. As the results of this experiment, presented in the Table, reveal, UVL-treated skin was unable to promote sensitization when DNFB was applied to that skin in an immunogenic manner. Incandescent light exposure had no comparable effect; these animals displayed strong contact hypersensitivity to DNFB when ear challenged. The circumstantial link between surface density of Langerhans cells and ability of skin to permit sensitization to DNFB was strengthened by a time course experiment in which UVL-treated skin was allowed to recover; at periodic intervals during the recovery period, UVL-treated skin was painted with DNFB in order to determine whether sensitization could be achieved. As can be seen by the results presented in Fig 1, there was a parallel association between the

Sensitization to DNFB: Role of Langerhans cell density

Skin painting site	Langerhans cell density (cells/mm^2)	Ear swelling response ($\times 10^{-4}$ ± SEM)
Abdominal body wall	770 ± 120	52 ± 2.1
Dorsal body wall	760 ± 150	50 ± 1.9
UVL-treated abdominal body wall	50 ± 30	15 ± 0.6
Tail	110 ± 15	16 ± 0.7
None (Negative control)		10 ± 0.6

Mice received 2 applications of 25 μl 0.5% DNFB on days 0 and 1. Ears were challenged with 20 μl of 0.2% DNFB on day 5. Swelling measured on day 6 and 7.

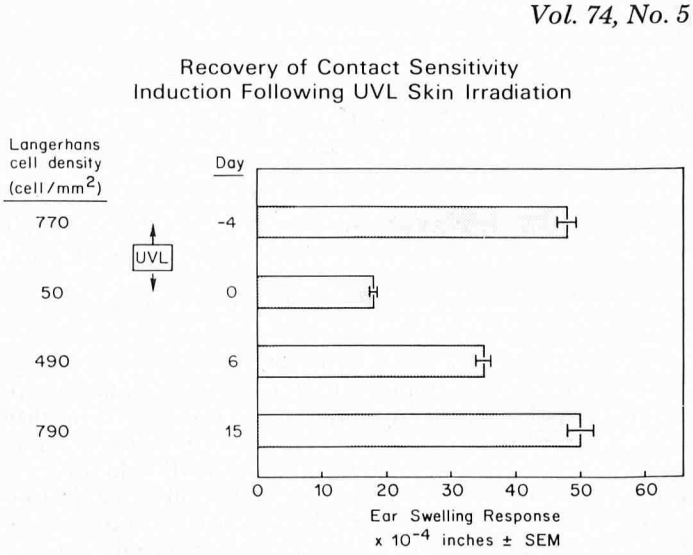

FIG 1. Panels of mice were UVL treated for 4 days. Skin was assayed for Langerhans cell density on days −4, 0, 6 and 15. Panels of mice were painted with 0.5% DNFB through UVL-treated skin on day 0, 6 or 15. Each panel was ear challenged 5 days after completion of skin sensitizing regimen. *Bars* represent mean ear swelling for groups of 5 mice ± 1 SEM.

quantitative return of Langerhans cell density toward normal and increasing capacity of that skin to permit DNFB sensitization. By 15 days after completion of UVL treatments, Langerhans cell density and morphology was normal, and this recovered skin permitted the development of normal sensitization to DNFB. Thus, skin depleted of Langerhans cells by UVL treatment no longer provides an appropriate medium to promote sensitization to locally painted DNFB.

Does Murine Tail Skin Permit Sensitization to DNFB?

The average Langerhans cell surface density in tail skin is considerably less than that of body wall skin; moreover, there is a maldistribution of Langerhans cells within tail epidermis: they are located at the periphery of tail skin scales, but are not present within the scale epidermis itself [18].

Panels of mice were painted on body wall skin or tail skin twice at 24-hr intervals with 0.5% DNFB in carrier. Five days later each panel was challenged on ear skin with 0.2% DNFB. The results are presented in the Table. Tail skin proved to be an inadequate surface through which to induce DNFB contact hypersensitivity. The amount of ear swelling that developed in tail skin-painted mice was only 32% that which developed in animals painted with DNFB on body wall skin, an amount not appreciably greater than the swelling that developed in negative control ears (20%). Tail skin shares with UVL-treated body wall skin the common property of low density of Langerhans cells; when DNFB is painted on either, sensitization fails to take place.

Does exposure to DNFB through Langerhans Cell-Depleted Skin Influence Subsequent Sensitization Through Normal Skin?

As mentioned in the Introduction, presentation of haptens by extracutaneous routes generally fails to immunize, resulting in specific unresponsiveness instead. To test whether skin depleted and/or deficient in Langerhans cells might similarly compromise host immune responsiveness, panels of mice were first exposed to DNFB by painting tail skin or UVL-treated body wall skin by the conventional immunizing regimen. Fourteen days later each group of mice was subjected again to the immunizing regimen, except that this time the painting was done on normal body wall skin. The ears of these animals as well as appropriate positive and negative controls were challenged 5 days later with 0.2.% DNFB. The results are presented in Fig 2. Exposure to DNFB through Langerhans cell-poor skin

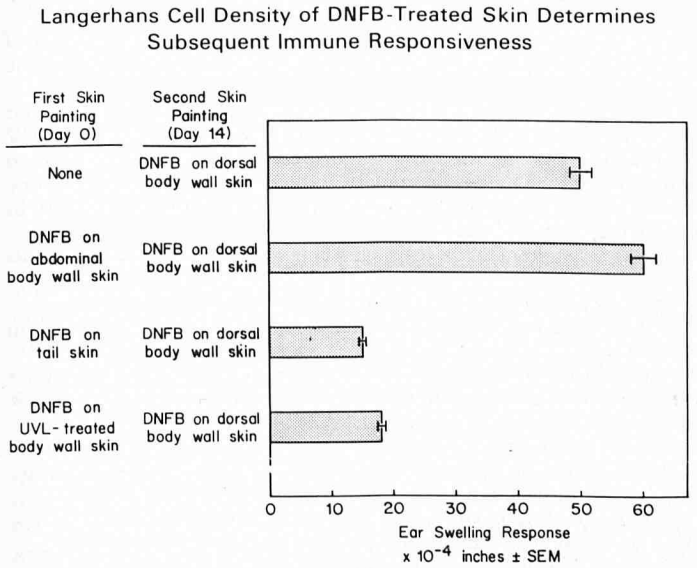

FIG 2. Panels of mice were first painted with 0.5% DNFB through normal abdominal wall skin, tail skin or UVL-treated skin. 14 days later, each panel (plus the positive control) was painted on dorsal body wall skin with 0.5% DNFB. Ear challenge was carried out 5 days later. *Bars* represent mean ear swelling for groups of 5 mice ± 1 SEM.

in each instance resulted in the development of profound unresponsiveness. Compared to negative controls, the % unresponsiveness achieved was 87.5% and 80% for tail skin and UVL-treated skin, respectively. In other experiments, the unresponsiveness was found to be highly specific in that DNFB-unresponsive animals were able to react vigorously to oxazalone [19].

Thus, while it is true that skin containing "sub-threshold" numbers of Langerhans cells fails to support sensitization to haptenic molecules, the immune system of animals so treated is not unperturbed. Instead, animals exposed to DNFB through skin deficient in Langerhans cells become profoundly unresponsive, as though they had received their initial exposure to the contactant by an extracutaneous route. We conclude that Langerhans cells provide skin with the unique and special attribute of both permitting and promoting the development of contact hypersensitivity.

DISCUSSION

The study of contact hypersensitivity is important from at least 2 standpoints. For the first—and especially since the system has been adapted in the last several years to permit its investigation among inbred strains of mice—contact hypersensitivity is an extraordinarily useful model to study the process by which T lymphocytes perceive antigen and are activated to a state of specific immunity. For the second, experimental contact hypersensitivity represents a reasonably faithful model of human disorders attributable to cell-mediated immunity directed at antigens that come in contact with skin. But contact hypersensitivity may prove to be a model system with more far reaching biologic implications. The derivatization of "self" determinants by highly reactive simple chemicals (haptens) is similar in immunologic terms to the modification of cell surfaces achieved by the expression of virus-specific antigens. These antigens are perceived by T lymphocytes in the context of "self" determinants encoded by the major histocompatibility complex [20]. And finally, the analogy may be extended further to the neoantigens expressed by malignant cells which may also induce T-cell mediated immunity in the context of MHC-related gene products. Assigning a pivotal role to epidermal Langerhans cells in the induction and perhaps elicitation of contact (delayed) hypersensitivity is therefore of both theoretical and practical importance.

Langerhans cells are a dynamic cell population. We are only beginning to probe experimentally the ways in which the numbers and physiologic functions of these cells can be deliberately or unwittingly perturbed. Exposure to actinic radiation may prove to be a crude method of altering the function of these important cells. And altering their function and numbers seems to have a profound effect on critical immune reactions. When Langerhans cells are present in skin in normal numbers and with normal function, they appear to play a sentinel role, equipping the cutaneous surface with an elaborate network of interlocking dendritic processes, prepared to capture foreign materials and render them strongly immunogenic. However, when Langerhans cells are absent from the skin or are significantly altered in physiologic properties, the network is breached and a surprisingly unorthodox face of the immune response is unmasked: exposure to foreign agents not only fails to sensitize, but evokes profound and specific immunologic unresponsiveness. If the ultimate physiologic role for Langerhans cells turns out to be that of converting potentially tolerogenic signals delivered to the skin into immunogenic ones, then skin devoid of these cells and their function may be vulnerable in several important ways.

It has already been reported that Langerhans cells form the major repository for certain pathogenic viruses that penetrate the cornified layer of epidermis [21]. Cutaneous surfaces deficient in Langerhans cells might be particularly susceptible to virus onslaught and unable to contain cell to cell spread of the agent. We have previously shown that corneal epidermis is utterly devoid of Langerhans cells [17] and consequently does not express cell surface determinants of the *I* region of *H-2* [22]. Perhaps the devastating and unrelenting infections of the cornea caused by herpes simplex virus infection owe their pernicious course to the absence of Langerhans cells from that tissue.

For purposes of discussion one can assume that malignant degeneration of keratinocytes and/or melanocytes in the skin is a capriciously random event. If the phenomenon of immunologic surveillance [23] is a physiologic mechanism by which the immune system rids the body of neoplastic cells then the strong clinical association [24–26] between prolonged exposure to actinic radiation and markedly increased incidence of skin cancer may also be linked through Langerhans cells. It is attractive to suppose that repeated exposure to UVL produces a chronic deficiency of epidermal Langerhans cells. When the inevitable neopastic cell or cluster of cells develops within epidermis, there is no sentinel to accept, process and present "neoantigens" to the central immunologic machinery as immunogens. Instead, the tumor specific antigens, in the absence of Langerhans cells (just as DNFB on UVL-treated skin), are able to deliver a tolerogenic signal, and thus procure an unmolested tenure for their illicit progency.

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DISCUSSION

GREEN: What happens to the efferent phase of contact reaction if you irradiate ears with UV light and then try to elicit a reaction in the ears?

STREILEIN: I wish I could tell you the answer to that. It is not that we haven't tried to do that experiment. It is not possible to deliver UV light to ear skin in the same exquisitely uniform way you can to abdominal skin. There is one report in the literature in which someone claims to have inhibited the expression of contact hypersensitivity in guinea pigs with UV light.

CLARK: Can you elicit a reaction when you paint DNFB on the tails of sensitized mice?

STREILEIN: We have not looked.

CLARK: Can you tolerize a sensitized mouse by painting DNFB on its tail?

STREILEIN: We've thought about doing that. It is somewhere on the list of priorities but not high enough. There is a bias, you know, that it is very hard to break through an established sensitized state.

CLARK: The reason for asking the question is probably obvious and that is—are you on the brink of showing us a way in which to tolerize people with rhus allergies?

STREILEIN: You mean people who are already sensitized? I do not think we are on the brink of that by any means.

CLAMAN: It is extraordinarily difficult to desensitize even when you have in your hands powerful tolerogens. The hope of desensitizing rhus sensitive patients is still over the horizon.

KATZ: Do you get any histological or gross morphologic changes with irradiation?

BERGSTRESSER: We have not looked carefully histologically, although acanthosis without necrosis is the most prominent change. Grossly, we get some scale and when we quadrupled the dose we produced ulceration after 4 days. After the full series of exposures we are near toxicity.

TIGALAAR: You showed us that the painting of ATP-ase depleted skin sites is not an immunologically null event and that the antigen is viewed as a tolerogenic stimulus by the animal in terms of its response to a second application of allergen. But is the poor response by such an animal also diminished when compared to animals skin painted both times on normal skin? I do not think you showed us that particular relevant control group.

STREILEIN: One of the controls we did, of course, was simply to paint animals on Day 1 and 2 and then on Day 14 and 15 and those animals respond well after the 14, 15 painting so there's nothing special about waiting that 2-week interval.

EDELSON: Since ultraviolet B, in significant amounts, increases keratinocyte disorganization and percutaneous absorption, your results need to be interpreted in the context of UVB-induced keratinocyte damage. Increased percutaneous absorption speaks in favor of a probe for an intra-epidermal cell in induction of sensitivity to the hapten. Could you comment on the potential relevance of these factors?

STREILEIN: We are aware that we are changing the barrier by UV light treatment. We are also aware that the barrier is probably different in tail scales. And, really, we have no better than circumstantial evidence to link Langerhans cell numbers to the failure to sensitize and to the unresponsiveness. I am not making any claims beyond that.

LEVIS: You have shown that you can block a primary sensitization to DNFB. What about a rechallenge?

STREILEIN: Well, we have not achieved Langerhans cell depletion with UV light sufficient to make us suspect that it would be worth doing the experiment yet.